Studying the Effects of Dilantin on DNA Polymerase Delta RNA Expression in Second Cell Cycle Preimplantation Mouse Embryos: Primer Design and Optimization for RT-PCR

An Honors Thesis (HONR 499)

By

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Abstract

Dilantin (DPH) is the most commonly prescribed anticonvulsant drug used today, but it is also known to be a human teratogen. Previous studies on the effects of DPH using NSA mice have revealed detailed information on the specific mechanisms of embryo development that are directly affected by DPH potentially causing the collection of fetal abnormalities termed fetal hydantoin syndrome (FHS). It has been shown that the cell cycle is deregulated in preimplantation mouse embryos treated with DPH by altering the expression of cyclin A. Even more, the DNA polymerase δ (Pol δ) protein catalytic subunit has been shown to have a decreased expression contributing to delayed DNA synthesis in 2-cell mouse embryos. This study continues the analysis of the DNA Pol δ subunit by beginning to examine the effect DPH has on the expression of Pol δ mRNA. This would explain whether Pol δ protein levels are being affected at the level of mRNA expression or later on at protein synthesis or a period beyond that. RT-PCR, gel electrophoresis, and ethidium bromide UV-fluorescence imaging were used as tools to examine the relative levels of expression of Pol δ mRNA of DPH and NaOH treated 2-cell mouse embryos at G1 and S phase of the second cell cycle. The results of this study were inconclusive in terms of determining the effect of Dilantin on Pol δ mRNA expression. However, the proper primers for Pol δ were designed, and the concentrations of $MgSO_4$ and the primers were both optimized at 2mM and 0.125mM respectably. The next step would be to isolate more embryos and run reactions at these optimal levels to determine the effect of DPH on Pol δ mRNA expression.

Acknowledgments

I want to thank Dr. Clare Chatot for welcoming me into her lab when no other research professor in the Biology or Chemistry departments would. She has been an awesome advisor for me during my entire time here at Ball State and especially helpful and supportive during this thesis work.

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Project Analysis Statement

This project has taken place during a very interesting pivot in my college career. Over the past 6 months, my goals have opened back up from the medical school tunnel vision I was experiencing. It is no longer clear to me if I will be going to medical school and preparing for work as a physician or getting a viticulture master's degree and working a grape farm and winery. Fortunately, should I pursue a vintner career instead of pursuing medical school, experience working in a biology research lab will still be advantageous.

The idea for this project was simply a product of necessity. During my entire time here at Ball State I have been doing anything and everything I can to make myself competitive for a spot in any medical school that would accept me. One of these things was getting involved with a research lab. So during the spring of sophomore year after being rejected from the chemistry department's summer research program, I contacted every biology professor with a research lab and Dr. Chatot responded with an invitation to get involved with her lab. Her lab was exploring the effects of an anti-seizure drug, Dilantin, on two things: sperm/egg interactions, and fertilized embryo development. I chose to work on the fertilized embryo project. A previous master's student who had completed her thesis in this lab essentially paved the way for the inquiry I would be making with my work. To prepare for this work, I was required to be certified in handling animals in a scientific research setting, so I spent that summer acquiring this certification through the collaborative institutional training initiative, CITI. The next step

of preparation was studying the mouse development in more detail as well as the details of designing and conducting a proper PCR protocol. Dr. Chatot taught me how to find PCR primer pairs specific to our species and target gene on a massive database. The last background work to be completed was my literature review. This task required me to review a large number of research articles pertaining to this work. Fortunately, the previous papers coming from this lab provided me with a good place to start.

What the completion of this project during this luminous time has taught me is to see things through no matter the changes in motivation. With this project being the most integrating of different bodies of knowledge that I have ever had to do, I feel this project helped me gain confidence in myself and in the knowledge and skills I have obtained during my time at Ball State.

INTRODUCTION

Dilantin is one of several-marketed antiepileptic drugs used in the treatment of seizures and several behavioral disorders. Women suffering from seizures who are being treated with antiepileptic drugs are advised to continue their treatment during pregnancy. Antiepileptic drugs are known to cause a variety of birth defects in the children of mothers taking these drugs during pregnancy. The collection of defects related to the use of Dilantin is called Fetal Hydantoin Syndrome (FHS) (Oguni and Osawa, 2004). FHS is characterized by multiple developmental and growth related abnormalities such as craniofacial abnormalities, cleft lip and palate, microcephaly, and limb deformities (Hanson and Smith, 1975). It is thought that the cause of these defects is related to the oxidative metabolic byproducts of these drugs produced *in vivo* by the fetus that disrupt many different mechanisms of embryogenesis (Wlodarczyk, 2012).

The metabolism of DPH has been studied in other mammalian species besides humans including rats (Dill et al., 1956), and mice (Chow & Fischer, 1982). The teratogenicity of DPH has been extensively studied in Mouse embryos (Sullivan and McElhatton, 1975), which have been the species of choice in the Chatot lab (Blosser and Chatot, 2003). In one study by Aide Cornielle-Dipre in the Chatot lab, it was discovered that Dilantin negatively effects the expression of the protein for DNA polymerase δ (Pol δ), a polymerase that functions in DNA replication along both the leading and lagging strand to elongate initiated DNA replication forks (Pavlov and Shcherbakova, 2010).

Using immunofluorescence with confocal microscopy, a 50% reduction in Pol δ protein was seen in the G1 phase of the second cell cycle in DPH treated embryos compared with NaOH vehicle control embryos. Interestingly there was no observed Pol δ protein reduction in 2-cell stage S phase DPH treated mouse embryos (Cornielle-Dipre Master's Thesis, 2010). In another study by Tolliver in the Chatot lab (Tolliver Master's Thesis, 2013), it was shown that DPH negatively affected the rate of DNA synthesis. The rate of synthesis was slowed in DPH treated embryos, which was measured at different time points. In a kinetics study, there was an 85% reduction in DNA synthesis rate for the first 5 minutes in DPH treated 2-cell S phase embryos in comparison to NaOH vehicle controls, a 20% reduction in rate in the first ten minutes, and an overall 31% reduction in rate over the entire 40 minutes of data collection. It is thought that this effect on DNA synthesis is due to a reduction in the expression of cyclin A and Pol δ (Tolliver and Chatot, 2013). The observed DPH induced reduction in cyclin A could reduce the rate of Pol δ function since cyclin A is needed for progressive replication fork elongation while a reduction in Pol δ concentration would reduce the number of functional replication forks elongating at any given time (Bashir et al., 2000). The causes of the reduction in Pol δ concentration are unknown. There may be a disruption in the mRNA expression that leads to lower Pol δ protein production, or the disruption could be in the Pol δ protein production itself.

In order to examine the effects of Dilantin on Pol δ mRNA expression, this study used gene-specific primers in an RT-PCR reaction to amplify the Pol δ RNA present in

preimplantation mouse embryos treated with DPH or NaOH vehicle *in vivo*. Determining the levels of amplified RNA for Pol δ in embryos at G1 and S phases of the second cell cycle would allow for evaluation of the relationship between Dilantin induced reduction in Pol δ protein and Pol δ mRNA expression.

The hypothesis for this study is that Dilantin will reduce the mRNA expression of DNA polymerase δ during G-1 and S phase of the second cell cycle in pre-implantation mouse embryos thus altering the elongation phase of DNA synthesis. This work concentrated on the design and optimization of RT-PCR for amplifying Pol δ RNA in control liver tissue and in G1 NaOH control embryos.

LITERATURE REVIEW

Dilantin Metabolism and Fetal Hydantoin Syndrome

Anticonvulsant drugs are widely used among pregnant women with 1 out of every 200 women taking these drugs during their pregnancy. These drugs are known to be associated with birth defects. Dilantin (DPH) is the most widely used anticonvulsant drug. The pattern of birth defects associated with the use of DPH during pregnancy is termed Fetal Hydantoin Syndrome (FHS). FHS is characterized by craniofacial abnormalities, cleft lip and palate, microcephaly, limb deformities, neurological defects, deficiencies in vitamin K and D, and cardiac defects (Hanson and Smith, 1975). The rate of birth defects among babies born to mothers taking an anticonvulsant drug is 2 to 3 times higher than the normal rate across the United States (Fitzgerald, 2004).

The teratogenic effects of DPH are understood to be a result of the metabolic byproducts that are formed during the bioactivation of DPH (Figure 1a) (Buehler et al., 1990). The most important byproduct is an arene oxide (Figure 1b) that is produced by the cytochrome P-450 monooxygenase pathway (Strickler et al., 1985). This arene oxide is an epoxide, which is an especially reactive compound due to its oxygen bridge. The oxygen bridge allows for covalent binding to embryonic macromolecules resulting in damage to DNA, RNA, and protein, which leads to a high risk of development abnormalities.

There is an enzyme, epoxide hydrolase, that can inactivate the reactive epoxide via hydrolysis making the byproducts, p-Hydroxy Phenytoin, m-Hydroxy Phenytoin, and Phenytoin Dihydrodiol soluble and removable from the body via the kidneys. It has been proposed that there are two alleles for the gene that encodes the epoxide hydrolase enzyme, fast and slow. The risk of developmental abnormalities depends on the fetus inheriting two slow isoforms resulting in an inability to quickly remove the epoxide before damage is done (Buehler et al., 1990) resulting in teratogenesis.

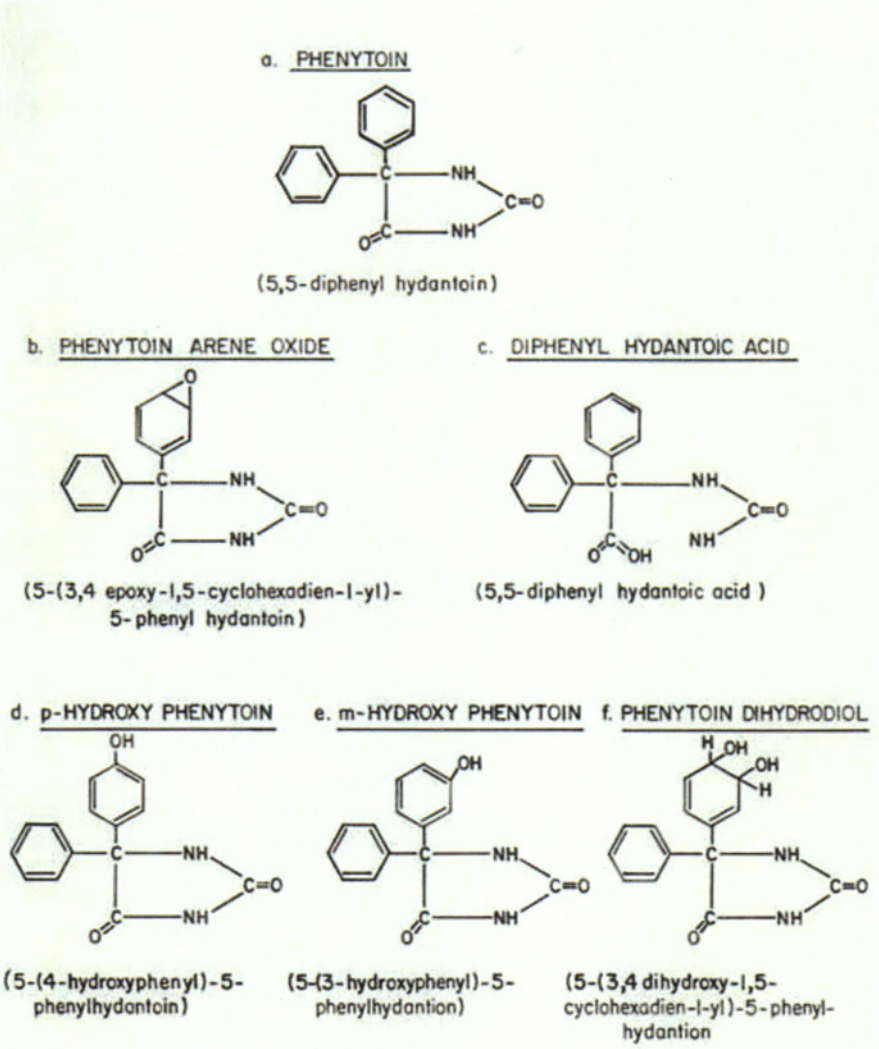

Figure 1. 2-D Chemical structure of DPH and metabolic by-products. Cytochrome P450 metabolizes **a**. phenytoin into **b**. phenytoin arene oxide or **c**. water-soluble diphenyl hydantoic acid. Epoxide hydrolase breaks down the phenytoin arene oxide to **d**. p-hydroxy phenytoin, **e**. m-hydroxy phenytoin or **f**. phenytoin dihydrodiol. (Chatot lab)

Preimplantation Mouse Embryo Development

Preimplantation mouse embryo development starts at fertilization of the mouse embryo in the ampulla of the oviduct (Sakkas and Vassalli, 2008). After fertilization, the embryo does not implant for four days while it travels down the oviduct to reach the uterus where it implants. The fertilized embryo goes from 1-cell to 32-cells during the first 3 and half days of this 4-day journey. The 1-cell embryo (Figure 2A) spends about 24 hours in the cell cycle, G1 is 3-8 hours long, S phase is 6 hours, and G2/M phase is 6 hours long (Krishna and Generosa, 1977). In the second cell cycle (2-cell embryo) (Figure 2B), G1 is 1.3 hours, S phase is 6.1 hours and 15.4 hours for G2/M phase for a total of 22.8 hours (Sawicki et al., 1978). During the middle of the 2-cell stage, the zygotic genome is activated switching the control of protein synthesis from the mother to the embryo (Piko and Clegg, 1982). The switch to embryo-controlled development makes the second cell cycle longer than a normal cell cycle, and can extend as long as 30 hours (Sakkas and Vassalli, 2008). During the 8-cell stage, the cell junctions become more integrated and boundaries disappear in a process called compaction (Figure 2C). At the 16-cell stage, the distinction is made between inner and outer cells forming the morula (Figure 2D). These populations expand during the blastocyst stage as the embryo prepares to implant in the uterus. The inner cells will become the fetus while the outer cells will integrate with the uterus forming the chorionic portion of the placenta (Gilbert, 2010). The current study will focus on the 2-cell stage (Figure 2 B) of development in G1 and S phases.

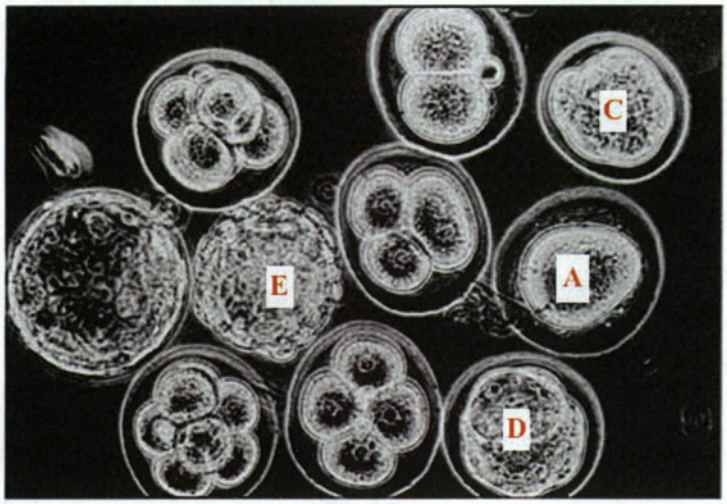

Figure 2: Preimplantation Mouse Embryo Developmental Stages. One-cell stage **A.**, Two-cell stage **B.**, Post-8 cell stage **C.**, Morula stage **D.**, Blastocyst **E.**

DNA Polymerases

DNA polymerases are enzymes that maintain the genome by performing DNA replication during cell division, by repairing damage to new DNA as well as bypassing damaged DNA during synthesis. All DNA polymerases share the same conformational structure similar to a human right hand with a palm, thumb, and fingers (Hübscher et al., 2002). There are many different types of DNA polymerases that are grouped into families based on their sequence and structure similarities. Polymerases α, δ, and ε make up the B family because they are all eukaryotic enzymes that function in DNA replication. Besides Pol δ, the other polymerases have their own role to play in DNA replication such as polymerase α that initiates the replication forks and polymerase ε that is traditionally thought to elongate the leading strand. These enzymes are each

made of a large subunit with several smaller subunits that function to maintain the large subunit.

Pol δ is made of four subunits. The p125 subunit is the larger catalytic subunit as seen in Figure 3. Then there are the p50, p66, and p12 subunits that all have their own role. The p50 subunit functions in maintaining the structure of the p125 subunit and stabilizes the p66 subunit. The p66 subunit functions in proliferating cell nuclear antigen interaction. This subunit is associated with regulating error-prone trans-lesion synthesis. The p12 subunit functions in protein-to-protein interactions during replication, but the mechanisms are not well understood (Pavlov and Shcherbakova, 2010).

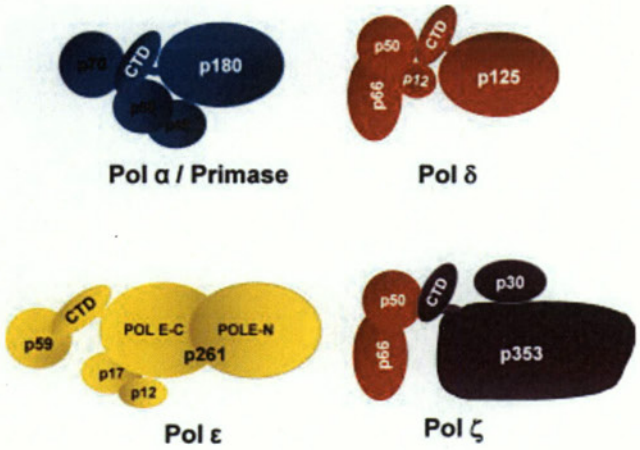

Figure 3. DNA polymerases α, δ, ε, and ζ subunit structures
(From Waisertreiger et al., 2012)

The role of Pol δ is to elongate and coordinate the synthesis along both leading and lagging strands of DNA from the RNA primer. This understanding of Pol δ changes the traditional replication fork model to an alternate model as seen in Figure 4. In the traditional standard fork model, Pol δ elongates along the lagging strand via Okazaki

fragments while polymerase ε, (Pol ε) elongates along the leading strand. It has been shown in yeast that when Pol ε is damaged, replication can still continue, but when Pol δ is damaged, replication is stopped. This was thought to indicate that Pol δ was taking over for Pol ε when it could not properly function. Further experimentation revealed that Pol δ more likely elongates along both strands normally rather than just taking over the leading strand elongation when Pol ε is failing. (Pavlov et Shcherbakova, 2010).

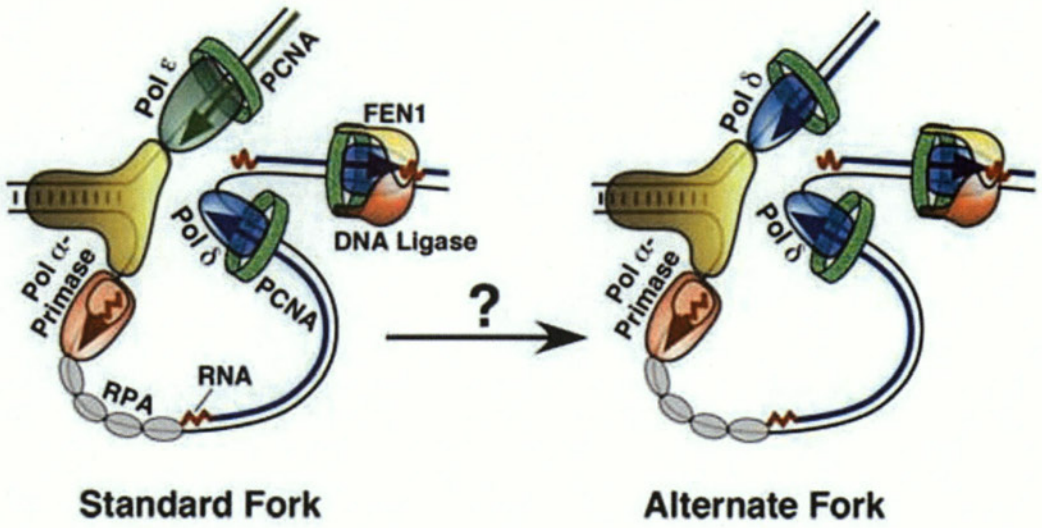

Figure 4. Representations of the traditional (Standard Fork) and the new model (Alternate Fork) of DNA replication. (From Burgers, 2009)

Significance

This study will add to the growing understanding of the specific mechanisms behind the teratogenic effects of anticonvulsant drugs by defining conditions to evaluate potential effects of DPH on Pol δ RNA expression, Better understanding of the functions disrupted by DPH in embryos could potentially aid in the development of better anticonvulsant drugs in the future that do not place pregnant women at as high of a risk of having children with birth defects.

MATERIALS & METHODS

DNA Polymerase δ is the enzyme used for the elongation of DNA during the replication process. Prior studies in the Chatot Lab have indicated that the protein for this DNA Polymerase is reduced in the mouse embryo during second cell cycle G-1 phase but not in S phase. This reduction could be due to reductions in mRNA concentration or in protein synthesis levels or to an increase in protein degradation. This study will address the question of whether DPH treatment alters levels of mRNA for the catalytic subunit of DNA Polymerase δ.

The goals of this study were to:

1. Design primers to allow for the amplification of mRNA for DNA Polymerase δ.
2. Optimize conditions using mouse liver extract to allow the use of the primers in an RT-PCR reaction to amplify mRNA for DNA Polymerase δ.
3. Use RT-PCR to determine if treatment with DPH alters the expression of mRNA for DNA Polymerase δ in embryos isolated at G1 and S phase of the second cell cycle in comparison to NaOH vehicle controls.

Primer Design

Using the NCBI primer-BLAST tool, several primers were designed to be used for amplifying the POLD1 gene that codes for the expression of the DNA polymerase δ p125 catalytic subunit. The primer set that was chosen to be used for the experiment was

determined based on specificity for the POLD1 gene. Primers with especially high specificity would lower the chances of getting contaminating non-specific DNA/RNA amplification and primer dimers. For POLD1, the 5' oligonucleotide sequence was 5'-TACCTCATCTCTCGGGCACA-3' and the 3' sequence was 3'-CTGTGCTGAACGTCCTCCTT-5'. The primers were chosen for their optimal GC content, which was 55%; their Tm values of 60.03 and 59.97 °C; their low self-complementarity scores of 2.00 and 4.00 respectively and the lack of 3' complementarity. To avoid unnecessary mismatches, GC content should remain between 40-60%, Tm values should be close together and self-complementarity low. Finally, there were only 2 other gene targets for these primers; a skeletal muscle myosin heavy chain variant and a myc induced nuclear antigen gene with product sizes of 1961 and 1296 bp respectively. Both are unlikely to be expressed in the 2-cell embryo.

Liver RNA Isolation

To ensure the selected primers amplified the target gene for polymerase δ, a positive control RNA was made using RNA isolated from liver tissue from female NSA mice using a Promega SV-Total RNA isolation system. The mice were sacrificed via cervical dislocation and portions of liver tissue were removed and placed in petri dishes containing 10X PBS. The tissue was blotted and weighed and 150 mg was placed in a tissue homogenizer with a Teflon pestle with the addition of 1300 µl of RNA lysis buffer, which denatures proteins including RNases that would otherwise degrade the RNA to be

isolated. After homogenizing the tissue, 175 µL of the solution was aliquoted into each of 4 fresh microcentrifuge tubes with the addition of 350 µL of dilution buffer to precipitate cellular debris and dissolve the RNA. After inverting the tubes several times, the tubes were heated at 70 °C for 3 minutes. The tubes were then centrifuged for 10 minutes at 14,000 X g to collect the precipitated debris into a pellet. The supernatant in each tube containing DNA and RNA was removed and placed in fresh tubes with the addition of 200 µL of 95% EtOH to precipitate the nucleic acids out of solution. After inverting the tubes several times, the tubes were transferred to fresh spin columns and centrifuged for 1 minute at 14,000 X g to trap the precipitated DNA and RNA in the basket matrix. After discarding the collection tube liquid, 600 µL of RNA wash solution was added to the spin baskets and spun down for 1 minute at 14,000 X g. The baskets were then treated with 50 µL of DNase solution for 15 minutes to break down any contaminating DNA before the addition of 200 µL of DNase stop solution to each basket and spun down for 1 minute at 14,000 X g to remove the dissolved degraded DNA from the column matrix. In order to purify the column of any remaining salts, proteins, and cellular debris a series of washes was performed starting with 600 µL of RNA wash solution being added and baskets being spun down for 1 minute at 14,000 X g. The collection tubes were emptied, and an additional 250 µL of RNA wash solution was added to the baskets and spun down for 2 minutes at 14,000 X g to remove the maximum amount of wash buffer. The baskets were transferred to new collection tubes where 100 µL of Nuclease-Free water was added and spun down for 1 minute at 14,000

X g to elute the RNA from the matrix into solution. The baskets were discarded and the RNA solutions were combined in a fresh microcentrifuge tube. A 1:100 dilution of sample was made from a portion of the RNA solution and analyzed in a spectrophotometer obtaining O.D. 260/280 readings to determine concentration and purity of the isolated RNA. The main solution was aliquoted into several PCR tubes for individual RT-PCR reactions and stored at -80 ^{o}C.

Embryo Isolation

Female NSA mice (internal breeding colony; original stock from Envigo, Indianapolis, IN) were superovulated with 10 IU of Pregnant Mare Serum Gonadotrophin (PMS) followed 48 hours later with 5 IU of human Chorionic Gonadotrophin (hCG). Females were mated immediately following hCG injection with B_6SJLF_1/J males (Jackson Laboratories, Bar Harbor, ME). Pregnant female NSA mice were treated with 55 mg/kg Dilantin dissolved in 0.001 N NaOH (dissolved to give the 55 mg/kg dose in 0.1 ml per 10 g of body weight) or with a comparable volume per body weight of 0.001 N NaOH as the vehicle control by intraperitoneal injection at 9:00 AM on the morning following mating.

NSA pregnant female mice were sacrificed by cervical dislocation (BSU approved IACUC protocol # 91855) at time points that would ensure the collection of embryos at G1 and S phase of the second cell cycle; i.e. 11 PM of the day after mating for 2-cell G1 and 3 AM of the day following mating for 2-cell S phases. If embryos had not yet

reached the 2-cell stage at the first isolation time point, the embryos were saved as 1-cell G2/M embryos. For embryo isolation, the oviducts were removed from the mice, washed in 0.9% saline, transferred to Hank Balanced Salt Solution with BSA (HBSS+BSA) and the embryos were flushed from the oviduct. Embryos were washed 3 times in HBSS +BSA and transferred to CZB medium without glucose. Embryos at G2/M 1-cell, G1 2-cell or S 2-cell phases were each transferred in random groups of 10 to 0.5 mL PCR tubes in 2 µl of CZB medium. One microliter of RNasin was added to each tube to prevent RNA degradation in storage and 10 µL of nuclease free water was added to lyse the embryo cells. Aliquots were frozen at -80 °C until use in RT-PCR.

RT-PCR Protocol for Embryo and Liver RNA

RT-PCR reactions were conducted on samples of Liver RNA and lysed mouse embryos using the Promega Access RT-PCR kit. Polymerase δ and GAPDH mRNA sequences were amplified. Due to a consistent expression of GAPDH in all mouse tissue, these sequences were amplified for an endogenous control.

To ensure optimal amplification of the target sequences and reduction of primer dimerization, several optimization experiments were conducted on both the liver tissue and mouse embryos. A magnesium curve experiment was done for both liver and embryo samples to determine optimal levels of $MgSO_4$ to use in the RT-PCR reactions for both samples using the POLD1 designed primer. Primer curve experiments were also completed in the same manner to determine the optimal primer concentration to use in

the RT-PCR reactions. GAPDH primers were run in the same reaction tubes with the POLD1 primers as a positive control. These primers were run through an optimization curve also, but the band sizes for the GAPDH (289 bp) and POLD1 (263 bp) primers were too similar to differentiate. GAPDH primers were then run in their own reaction tubes with each POLD1 experiment using the same reaction mix to maintain the necessary positive control without interfering with the analysis of the POLD1 band patterns. For GAPDH primers, the 5' sequence was 5'-GCATGGCCTTCCGTGTTCCT-3' and the 3' sequence was 3'-CCCTGTTGCTGTAGCCGTATTCAT-5'.

The positive control tubes contained 10 µL of AMV/Tfl 5X reaction buffer, 1 µL dNTP mix, 4 µL $MgSO_4$, 1 µg mouse liver RNA, and 1.0 µL of AMV reverse transcriptase and Tfl polymerase. The Tfl polymerase was added to each tube after the PCR machine reached the 94 °C denaturation temperature constituting a "Hot-Start" to minimize primer dimerization. In the negative control tube, nuclease-free water was added in place of the RNA sample.

The reaction tubes containing the mouse embryos differed in respect to the amounts of primers and Nuclease-free water added, but everything else was maintained the same including the total reaction volume of 50 µL.

The parameters for the RT-PCR reactions were as follows. 1st strand of cDNA synthesis: 1 cycle of 45 °C for 45 minutes to 94 °C for 2 minutes. Once the temperature reached 94 °C, the machine was paused for addition of the Tfl polymerase. The tubes were promptly spun down and placed back into the machine where the cycle was

resumed at 94 °C for 2 minutes. Amplification: 40 cycles of 94 °C for 30 seconds to 60 °C for 1 minute to 68 °C for 2 minutes. Completion: 1 cycle of 68 °C for 7 minutes. The machine then dropped to 4 °C where it remained until the tubes were removed for agarose gel electrophoresis.

Agarose Gel Electrophoresis

An agarose gel electrophoretic analysis was conducted using a 4% agarose gel in a 1X TBE (89 mM Tris (pH 7.6), 89 mM Boric acid, 2 mM EDTA) buffer with the addition of Ethidium Bromide at a ratio of 5 µL/500ml 1X TBE. Fifteen microliters of each RT-PCR sample was placed in a new PCR tube with the addition of 2 µL of 10X loading buffer (65% (w/v) Sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.3% (w/v) Bromophenol Blue). Nine microliters of this solution was loaded into the gel. For the ladder sample, 2 µL of 100bp Promega ladder was placed in a fresh PCR tube with 2 µL 10X loading buffer and 6 µL of 1X TBE. Nine microliters of this sample was loaded in the same manner. The gels ran at 62 volts for approximately 4 hours. Using a Biorad Gel imaging machine and Gel Doc™ XR software, the gels were visualized under UV light.

RESULTS/DISCUSSION

Due to difficulties with the treated mouse embryos, there were no results found on the effects of DPH on Pol δ mRNA expression. Although these results were not obtained, two of the three goals of this study were still met. Primers were designed properly to amplify the Pol δ mRNA. Optimization experiments were also successfully completed in both mouse liver and embryo samples.

Pol δ Primer optimization in Liver Tissue

The optimization of the Pol δ primer concentration was first done using a mouse liver sample. The proper band size for these primers was approximately 263 bp in length. The primer concentration can determine how much mRNA is amplified as well as how much background amplification occurs. The goal was to get the most liver Pol δ mRNA amplification with the least amount of background amplification. The results of this experiment showed that 0.125mM of the primers gave the best amplification with the least amount of background amplification as seen in Figure 5, lane 3. The background amplification was likely the result of primer dimers.

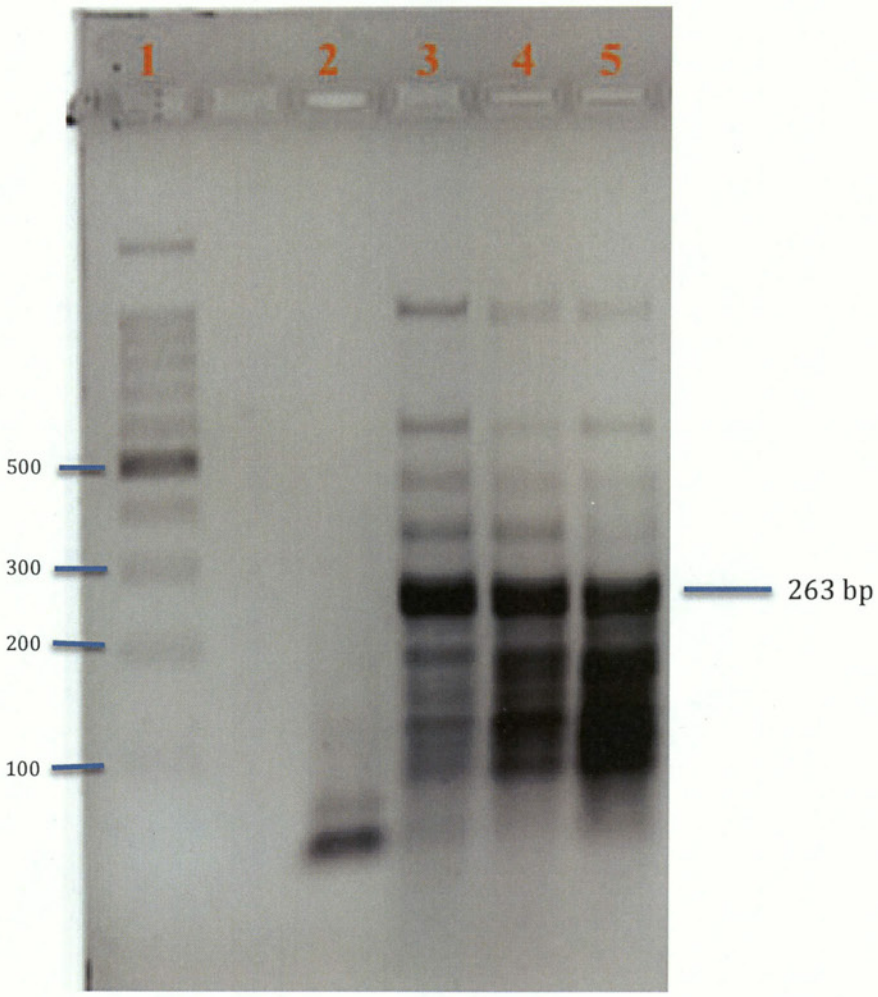

Figure 5: Effect of primer concentration on Pol δ mRNA expression in Mouse Liver. 1. 100bp ladder, **2.** Negative control, **3.** 0.125 mM Pol δ primers, **4.** 0.25 mM Pol δ primers, **5.** 0.5 mM Pol δ primers

Magnesium Sulfate Concentration Optimization in Liver Tissue

The other important concentration to optimize is the magnesium concentration since magnesium is essential for the binding of nucleotides to make cDNA and it determines how well the reverse transcriptase enzyme will work in making cDNA from the RNA template. The results showed that 2mM $MgSO_4$ was the concentration that provided the highest Pol δ amplification with the least amount of background amplification as seen in Figure 6, lane 4.

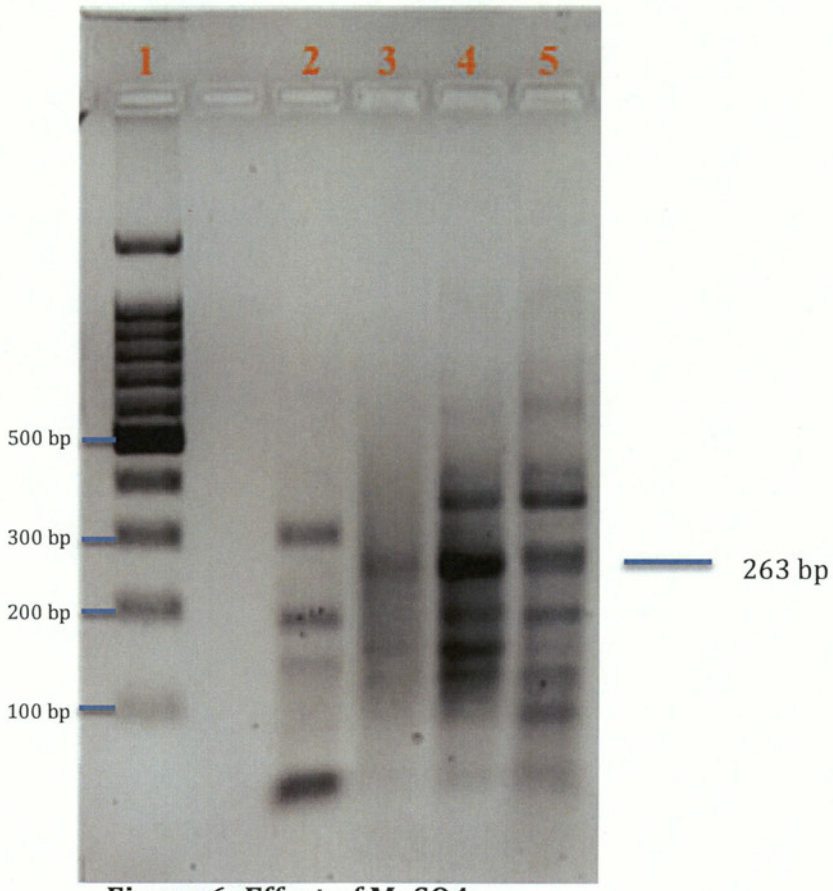

Figure 6: Effect of MgSO4 concentration on Pol δ mRNA expression in Mouse Liver. **1.** 100bp ladder, **2.** Negative control, **3.** 1 mM MgSO4, **4.** 2 mM MgSO4, **5.** 3 mM MgSO4

GAPDH & Pol δ Primer Concentration Optimization in Liver Tissue

For a positive control to ensure the PCR reaction was working properly and the bands being used to determine Pol δ expression were not just coincidentally sized DNA contaminants, GAPDH primers were optimized alongside the Pol δ primers. As seen in Figure 7, lane 4, GAPDH and the Pol δ primers produced DNA bands very similar in size. These experiments could not be properly interpreted with this similarity in band size, so the primers were optimized separately to make use of the GAPDH positive control without making the interpretation of the Pol δ mRNA expression impossible.

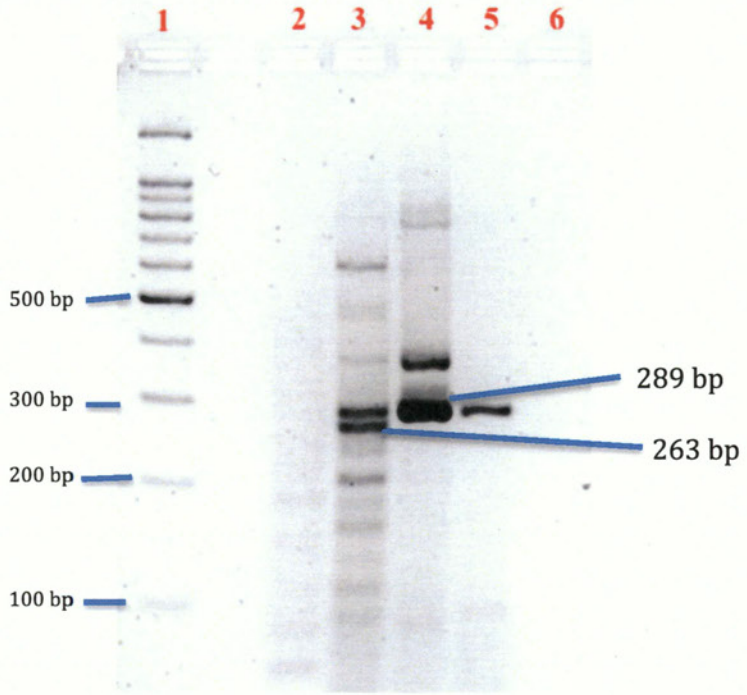

Figure 7: Effect of primer concentration on Pol δ mRNA/ GAPDH expression in Mouse Liver. **1.** 100bp ladder, **2.** Negative control, **3.** 1.25 mM Pol δ, **4.** 1.125 mM GAPDH, **5.** Pol δ primers & GAPDH, **6.** Pol δ primers & GAPDH w/o AMV-RT

Pol δ Primer Optimization in NaOH treated 2-cell G1 Embryos

Figure 8 shows the results of optimizing the Pol δ primers separately in 2-cell stage S phase NaOH vehicle embryos with the optimized magnesium concentration of 2mM.

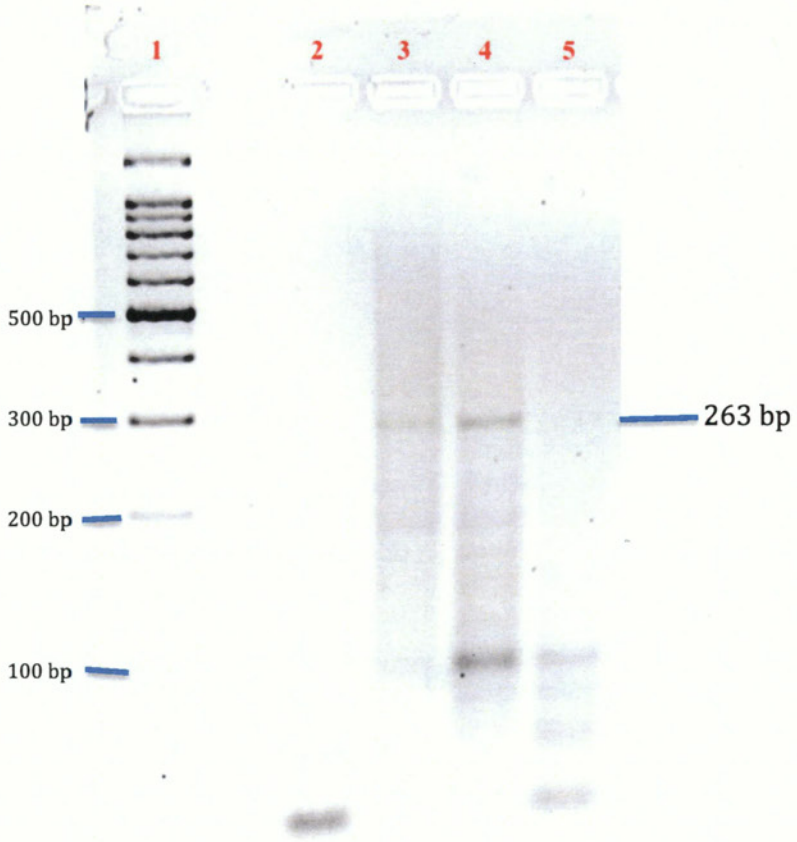

Figure 8: Effect of Pol δ primer concentration on Pol δ mRNA expression in NaOH treated Embryos. **1.** 100 bp ladder, **2.** Negative control, **3.** 0.125 mM Pol δ primers, **4.** 0.25 mM Pol δ primers, **5.** 0.5 mM Pol δ primers

Future Improvements

There were several issues with the proper execution of this experiment that could have led to error causing the issues obtaining viable results. The embryos used in this experiment were isolated between 4 and 6 months before they were used for the PCR procedures. This time gap could have allowed for degradation of the RNA. The freeze-thaw cycles help lyse the cell membranes to release the RNA into solution, so not enough quick cycles of this may have allowed RNA to remain blocked by the membrane from being present in the reaction. Another issue was with the electrophoresis buffer, which contained a much smaller amount of the ethidium bromide dye used to mark the bands for imaging than what has been used for previous experiments like this. So improvements would include using the isolated embryos soon after isolating them, increasing the ethidium bromide concentration in the electrophoresis buffer, and running the isolated embryos through one or two more freeze-thaw cycles. To improve upon this experiment to successfully determine the effects of Dilantin on Pol D mRNA expression, improvements in the isolation of embryos, the lysing of those embryos via freeze-thaw cycles, and the electrophoresis buffer may all aid in obtaining definitive results.

Conclusion

The presence of bands that are approximately 263bp with a low amount of background amplification indicates the successful amplification of the target gene, POLD-1. The primers were therefore designed and optimized properly. The resulting optimal Pol D primer concentration was 0.25 mM and the optimal magnesium sulfate concentration was 2 mM.

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COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COURSEWORK REQUIREMENTS REPORT*

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- **Name:** Peter Carnevale (ID: 4842216)
- **Email:** pecarnevale@bsu.edu
- **Institution Affiliation:** Ball State University (ID: 1568)
- **Institution Unit:** Biology

- **Curriculum Group:** Working with the IACUC
- **Course Learner Group:** Investigators, Staff and Students
- **Stage:** Stage 1 - Lab Animal Research
- **Description:** The CITI Basic Course in Laboratory Animal Welfare for Investigators, Staff and Students.

- **Report ID:** 16404625
- **Completion Date:** 06/18/2015
- **Expiration Date:** 06/17/2018
- **Minimum Passing:** 80
- **Reported Score*:** 100

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED	SCORE
Introduction to Working with the IACUC (ID:1807)	06/18/15	No Quiz
Working with the IACUC (ID:1808)	06/18/15	3/3 (100%)
Federal Mandates (ID:1809)	06/18/15	5/5 (100%)
The Veterinary Consultation (ID:1810)	06/18/15	1/1 (100%)
Getting Started (ID:1811)	06/18/15	5/5 (100%)
Alternatives (ID:1812)	06/18/15	8/8 (100%)
Avoiding Unnecessary Duplication (ID:1813)	06/18/15	1/1 (100%)
USDA Pain/Distress Categories (ID:1814)	06/18/15	8/8 (100%)
Endpoint Criteria (ID:1815)	06/18/15	2/2 (100%)
Surgery (ID:1816)	06/18/15	11/11 (100%)
Antibody Production (ID:1817)	06/18/15	No Quiz
Collecting Blood Samples (ID:1818)	06/18/15	3/3 (100%)
Personnel Training and Experience (ID:1819)	06/18/15	3/3 (100%)
Occupational Health and Safety (ID:1820)	06/18/15	1/1 (100%)
Using Hazardous and Toxic Agents in Animals (ID:1821)	06/18/15	5/5 (100%)
Housing Social Animals (ID:1822)	06/18/15	1/1 (100%)
Housing Rodents on Wire Floors (ID:1823)	06/18/15	1/1 (100%)
Dog Exercise (ID:1824)	06/18/15	1/1 (100%)
Primate Psychological Enrichment (ID:1825)	06/18/15	1/1 (100%)
Prolonged Restraint (ID:1826)	06/18/15	2/2 (100%)
Euthanasia (ID:1827)	06/18/15	5/5 (100%)
Using Human Patient Care Areas for Animal Research (ID:1828)	06/18/15	1/1 (100%)
Using Explosive Agents in the Animal Facility (ID:1829)	06/18/15	1/1 (100%)
Making Changes after You Receive Approval (ID:1830)	06/18/15	1/1 (100%)
Reporting Misuse, Mistreatment, or Non-Compliance (ID:1831)	06/18/15	No Quiz
Final Comments (ID:1832)	06/18/15	No Quiz

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COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COURSEWORK TRANSCRIPT REPORT**

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- **Institution Unit:** Biology

- **Curriculum Group:** Working with the IACUC
- **Course Learner Group:** Investigators, Staff and Students
- **Stage:** Stage 1 - Lab Animal Research
- **Description:** The CITI Basic Course in Laboratory Animal Welfare for Investigators, Staff and Students.

- **Report ID:** 16404625
- **Report Date:** 06/18/2015
- **Current Score**:** 100

REQUIRED, ELECTIVE, AND SUPPLEMENTAL MODULES	MOST RECENT	SCORE
Introduction to Working with the IACUC (ID:1807)	06/18/15	No Quiz
Working with the IACUC (ID:1808)	06/18/15	3/3 (100%)
Federal Mandates (ID:1809)	06/18/15	5/5 (100%)
The Veterinary Consultation (ID:1810)	06/18/15	1/1 (100%)
Getting Started (ID:1811)	06/18/15	5/5 (100%)
Alternatives (ID:1812)	06/18/15	8/8 (100%)
Avoiding Unnecessary Duplication (ID:1813)	06/18/15	1/1 (100%)
USDA Pain/Distress Categories (ID:1814)	06/18/15	8/8 (100%)
Endpoint Criteria (ID:1815)	06/18/15	2/2 (100%)
Surgery (ID:1816)	06/18/15	11/11 (100%)
Antibody Production (ID:1817)	06/18/15	No Quiz
Collecting Blood Samples (ID:1818)	06/18/15	3/3 (100%)
Personnel Training and Experience (ID:1819)	06/18/15	3/3 (100%)
Occupational Health and Safety (ID:1820)	06/18/15	1/1 (100%)
Using Hazardous and Toxic Agents in Animals (ID:1821)	06/18/15	5/5 (100%)
Housing Social Animals (ID:1822)	06/18/15	1/1 (100%)
Housing Rodents on Wire Floors (ID:1823)	06/18/15	1/1 (100%)
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Using Explosive Agents in the Animal Facility (ID:1829)	06/18/15	1/1 (100%)
Making Changes after You Receive Approval (ID:1830)	06/18/15	1/1 (100%)
Reporting Misuse, Mistreatment, or Non-Compliance (ID:1831)	06/18/15	No Quiz
Final Comments (ID:1832)	06/18/15	No Quiz

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COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COURSEWORK REQUIREMENTS REPORT*

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- **Institution Unit:** Biology

- **Curriculum Group:** Working with Mice in Research Settings
- **Course Learner Group:** Working with Mice in Research
- **Stage:** Stage 1 - Lab Animal Research

- **Report ID:** 16088238
- **Completion Date:** 05/28/2015
- **Expiration Date:** 05/27/2018
- **Minimum Passing:** 80
- **Reported Score*:** 87

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED	SCORE
Introduction to Working with Mice in Research Settings (ID:1933)	05/28/15	No Quiz
Research Mandates and Occupational Health Issues (ID:1934)	05/28/15	3/3 (100%)
Alternatives Searches, Humane Standards, Housing, and Acclimation and Quarantine (ID:1936)	05/28/15	2/2 (100%)
Detecting Pain and Distress, Genetics, and Biological Features (ID:1940)	05/28/15	3/3 (100%)
Injections, Blood Collection, and Antibody Production (ID:1943)	05/28/15	2/2 (100%)
Surgery, Supportive Care and Monitoring, Euthanasia, and References (ID:1946)	05/28/15	3/5 (60%)

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COURSEWORK TRANSCRIPT REPORT**

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- **Course Learner Group:** Working with Mice in Research
- **Stage:** Stage 1 - Lab Animal Research

- **Report ID:** 16088238
- **Report Date:** 05/28/2015
- **Current Score**:** 100

REQUIRED, ELECTIVE, AND SUPPLEMENTAL MODULES	MOST RECENT	SCORE
Introduction to Working with Mice in Research Settings (ID:1933)	05/28/15	No Quiz
Research Mandates and Occupational Health Issues (ID:1934)	05/28/15	3/3 (100%)
Alternatives Searches, Humane Standards, Housing, and Acclimation and Quarantine (ID:1936)	05/28/15	2/2 (100%)
Detecting Pain and Distress, Genetics, and Biological Features (ID:1940)	05/28/15	3/3 (100%)
Injections, Blood Collection, and Antibody Production (ID:1943)	05/28/15	2/2 (100%)
Surgery, Supportive Care and Monitoring, Euthanasia, and References (ID:1946)	05/28/15	5/5 (100%)

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- **Institution Affiliation:** Ball State University (ID: 1568)
- **Institution Unit:** Biology

- **Curriculum Group:** Working With Animals In Biomedical Research - Refresher Course
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - Lab Animal Research

- **Report ID:** 16088239
- **Completion Date:** 05/21/2015
- **Expiration Date:** 05/20/2017
- **Minimum Passing:** 80
- **Reported Score*:** 100

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED	SCORE
Introduction to Working with Animals In Biomedical Research - Refresher Course Modules (ID:2048)	05/21/15	1/1 (100%)
The Regulators (ID:2049)	05/21/15	2/2 (100%)
Where The Buck Stops (ID:2050)	05/21/15	3/3 (100%)
Yeah, But I'm From Missouri (The "Show Me" State) (ID:2051)	05/21/15	2/2 (100%)
To Be Or Not To Be (ID:2052)	05/21/15	1/1 (100%)
Something Old, Something New (ID:2053)	05/21/15	1/1 (100%)
The Right Person For The Right Job (ID:2054)	05/21/15	1/1 (100%)
There's No Place Like Home (ID:2055)	05/21/15	1/1 (100%)
This Will Hurt Me More Than It Will Hurt You (ID:2056)	05/21/15	1/1 (100%)
The Most Unkindest Cut of All (ID:2057)	05/21/15	3/3 (100%)
After The Dance (ID:2058)	05/21/15	2/2 (100%)
No Mas! No Mas! (ID:2059)	05/21/15	1/1 (100%)
Riding Off Into The Sunset (ID:2060)	05/21/15	1/1 (100%)
A Canary In The Mineshaft (ID:2061)	05/21/15	1/1 (100%)

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- **Stage:** Stage 1 - Lab Animal Research

- **Report ID:** 16088239
- **Report Date:** 05/22/2015
- **Current Score**:** 100

REQUIRED, ELECTIVE, AND SUPPLEMENTAL MODULES	MOST RECENT	SCORE
Introduction to Working with Animals In Biomedical Research - Refresher Course Modules (ID:2048)	05/21/15	1/1 (100%)
The Regulators (ID:2049)	05/21/15	2/2 (100%)
Where The Buck Stops (ID:2050)	05/21/15	3/3 (100%)
Yeah, But I'm From Missouri (The "Show Me" State) (ID:2051)	05/21/15	2/2 (100%)
To Be Or Not To Be (ID:2052)	05/21/15	1/1 (100%)
Something Old, Something New (ID:2053)	05/21/15	1/1 (100%)
The Right Person For The Right Job (ID:2054)	05/21/15	1/1 (100%)
There's No Place Like Home (ID:2055)	05/21/15	1/1 (100%)
This Will Hurt Me More Than It Will Hurt You (ID:2056)	05/21/15	1/1 (100%)
The Most Unkindest Cut of All (ID:2057)	05/21/15	3/3 (100%)
After The Dance (ID:2058)	05/21/15	2/2 (100%)
No Mas! No Mas! (ID:2059)	05/21/15	1/1 (100%)
Riding Off Into The Sunset (ID:2060)	05/21/15	1/1 (100%)
A Canary In The Mineshaft (ID:2061)	05/21/15	1/1 (100%)

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